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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

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Emulsan quantitation by Nile red quenching fluorescence assay

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Abstract A Nile red fluorescent technique to quantify 20–200 μg ml^{-1} of emulsan was developed. Nile red dissolved in DMSO showed an adsorption peak at 552 nm, and emission peak at 636 nm, with molar extinction coefficient of 19,600 cm^{-1} M^{-1}. Nile red fluorescence in DMSO was proportionally quenched by emulsan and the quenching was time-dependent. The assay was used to follow the production of emulsan by cultures of *Acinetobacter venetianus* RAG-1.

Introduction

Nile red, 9-diethylamino-5H-benzo[α]phenoxazime-5-one, fluorescence is strongly influenced by environmental conditions (Deye et al. 1990; Greenspan and Fowler 1985). These properties of Nile red allowed using the dye as a probe to determine the polarity of organic solvents, solvent mixtures, supercritical fluids, and ionic liquids (Deye et al 1990; Carmichael and Seddon 2000), to test the environment of zeolites, synthetic polymers, and liquid crystals (Greenspan and Fowler 1985; Choi et al. 1997). In microbiology, Nile red has been used to determine and quantify the synthesis of polyhydroxyalkanoates and wax esters in many Gram-negative bacteria (Gorenflo et al. 1999; Spiekermann et al. 1999). In other biological systems, Nile red has been used as a probe to determine the presence of neutral lipids, and hydrophobic proteins (Greenspan and Fowler 1985; Sackett and Wolf 1987).

Emulsifiers are amphiphilic molecules with the ability to disperse non-miscible liquids into a single phase. Emulsan, one of the most potent emulsifiers synthesized by *Acinetobacter venetianus* RAG-1 (formerly *A. calcoaceticus* RAG-1; Vaneechoute et al. 1999), is about 1×10^6 Da and composed of a trisaccharide repeat unit covalently linked with fatty acids (Rosenberg and Ron 2002). Potential industrial uses of emulsan range from bioremediation of heavy metals and oil recovery, to medical adjuvants in order to enhance humoral immunity (Panilaitis et al. 2002; Rosenberg and Ron 2002). The procedure currently used to quantify emulsan is based on measuring emulsification activity by incubating a sample of the polymer with *n*-octanol, and determining turbidity spectrophotometrically at 600 nm (Johri et al. 2002). The technique is not highly reproducible and also not sensitive below 1 mg ml^{-1} of emulsan, which is not useful from the point of view of emulsan biosynthesis during cell culture. Larges sample volumes and time requirements of the current assay are additional constraints.

The aim of the present study was to develop a simple and sensitive fluorescent assay to quantify emulsan based on the quenching of Nile red fluorescence by the emulsifier. The assay was evaluated during the production of emulsan by *A. venetianus* RAG-1.

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Materials and methods

A. venetianus RAG-1 (ATCC 31012) was cultivated on defined saline medium and cultivated as previously reported (Johri et al. 2002). After the 6th day, cultures were centrifuged (5,000 *g*, 30 min, 4°C) and washed with water twice. Supernatants were collected and filtered on carbon filter units (Whatman, N.J.). The filtered solution was collected and precipitated with acetone 60%, and kept overnight at –20°C. Precipitates were collected by centrifugation at 10,000 *g* for 45 min (4°C). The pellets con-

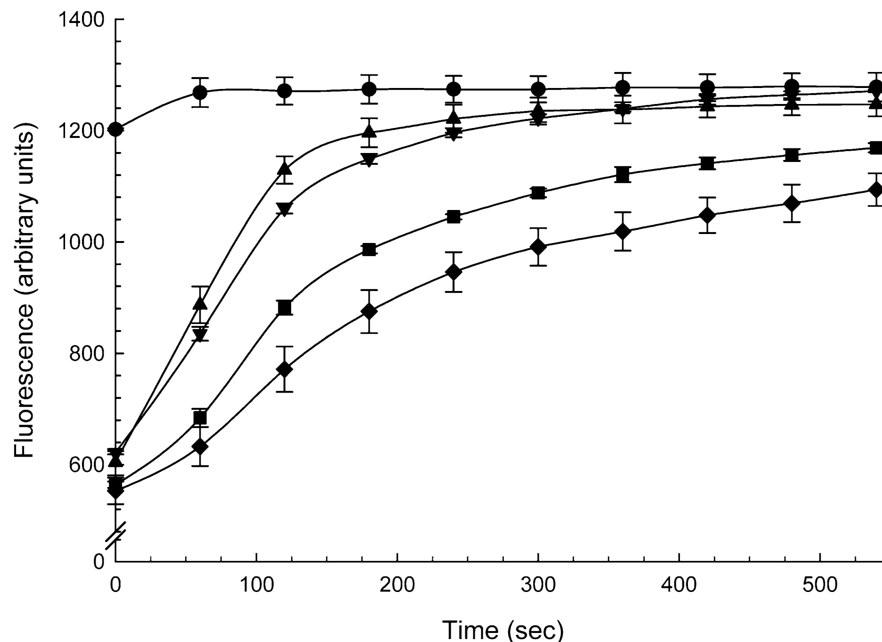

Fig. 1 Kinetics of Nile red fluorescence in the absence (•), or in the presence of emulsan: 20 μg ml^{-1} (▴), 67 μg ml^{-1} (▾), 100 μg ml^{-1} (▪), and 200 μg ml^{-1} (♦), respectively. Values are averages±SD (n=3–4)

taining emulsan were purified as described before (Johri et al. 2002).

Stock solution of Nile red (Sigma, Mo.) was prepared at 500 μg ml^{-1} in DMSO (Fisher, N.J.). Fluorescence experiments were carried out on Cary Eclipse (Varian, Calif.) or F4500 (Hitachi, Ill.) spectrofluorometers with 5 nm emission and excitation slit width at 25°C in a 1-ml path-light cuvette. One-milligram freeze dried samples of pure and crude emulsans from fermentation broths were dissolved in 1 ml DMSO.

Results

In previous reports Nile red fluorescence was used to detect properties of polyhydroxyalkanoic acids, and proteins, and the resulting fluorescence of the solution was attributed to the interaction between the hydrophobic domains of the molecules with the dye (Daban et al. 1991, Dutta et al. 1996; Gorenflo et al. 1999). However, no changes in the Nile red emission spectra in the presence of 1.0 mg ml^{-1} emulsan in water were found, likely because the fatty acid content in the emulsan molecule is about 15% weight.

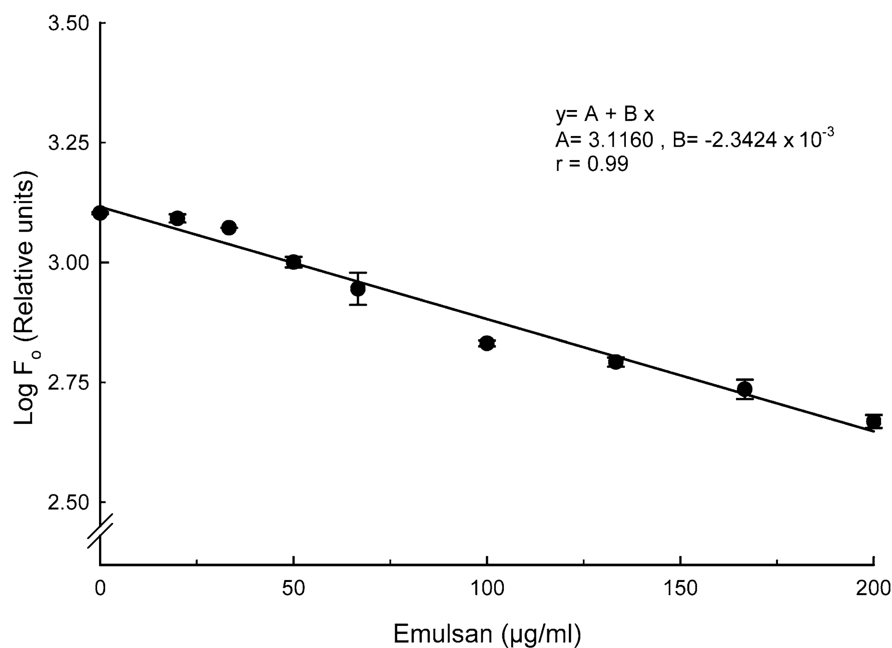

Fig. 2 Calibration curve of emulsan with Nile red. Data are averages±SD (n=3–4)

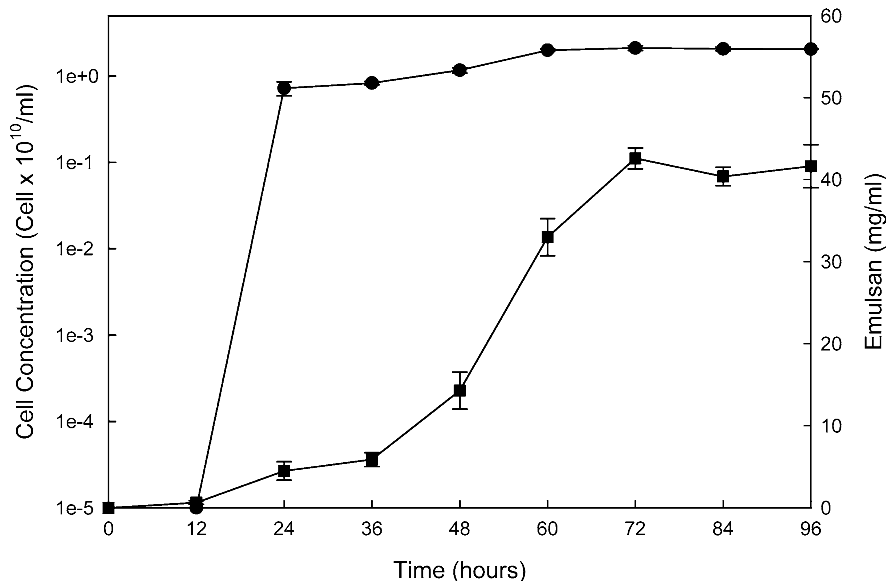

Fig. 3 Time-course of cell growth of *Acinetobacter venetianus* RAG-1 (•), and emulsan production detected by Nile red fluorescent technique (▪). Data are averages±SD (n=3)

Also, the solubility of Nile red in water is low (<1 μg ml^{-1}), and dye solutions in water are unstable with precipitates appearing after 2 weeks. However, Nile red has good solubility in many organic solvents (Deye et al. 1990). Emulsan solubility was tested in many organic solvents (data not shown), but the only solvent in which emulsan could be dissolved in amounts >1.0 mg ml^{-1} was DMSO.

Excitation and emission spectra for Nile red in DMSO presented only single major peaks at 552 nm and 636 nm, respectively (data not shown). The calculated molar extinction coefficient of Nile red in DMSO at 552 nm was 19,600 cm^{-1} M^{-1}. In addition, Nile red dissolved in DMSO is stable in the dark at 200 μg ml^{-1} and at room temperature for >1 year, an additional advantage of this system.

Addition of emulsan to Nile red solutions did not produce changes in maximum emission. However, considering the emulsifying properties of emulsan which substantively modify surface tension and interfacial tension in aqueous solution in the presence of oils and organic solvents (Zhang et al. 1999), it is possible to the predict transient changes in the fluorescence spectra of Nile red associated with the emulsifying activity. Different concentrations of Nile red were tested between 1.0 to 200 μg ml^{-1} in DMSO, and with or without emulsan (data not shown). The concentration of Nile red was adjusted to 20 μg ml^{-1} in order to quantify in the range of 20–200 μg ml^{-1} emulsan in 1.0 ml solution total volume. The kinetics of Nile red fluorescence quenching emission at 636 nm by increased emulsan concentration is shown in Fig. 1. A linear correlation between the changes of Nile red fluorescence with emulsan concentration at 5 min is shown in Fig. 2. However, changes in the reading time allowed the assay to be modified to an appropriate range and sensitivity. With our experimental conditions, this Nile red assay for emulsan is about 50 times more sensitive compared to the standard turbidimetric assay which is not sensitive below 1.0 mg ml^{-1} of emulsan. Another advantage of the Nile red assay is that requires <1 ml of sample from a culture compared to 7.5 ml sample for the turbidimetric assay.

The Nile red fluorescent assay was used to follow em ulsan production during 96 h in a batch culture of *A. venetianus* RAG-1. Before 36 h of culture, the turbidimetric technique did not detect emulsan in the supernatant. In contrast, using the Nile red fluorescent technique, emulsan was detected at 12 h of culture (Fig. 3).

Discussion

In conclusion, a new sensitive assay to quantify emulsan based on Nile red fluorescence quenching was developed. The disadvantage of this technique is that the sample must be dried and dissolved in neat organic solvent. The major advantages of the assay are high sensitivity, low sample volumes, and reduced reaction times. In addition, this technique can be extended to other types of bioemulsifiers and molecules able to interact with Nile red as this is an environment-sensitive fluorescent dye. This new technique should improve the ability to study bioemulsifier secretion by microbial systems in response to environmental factors, leading to new applications.

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